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Microbiological Research Authority
CAMR
Porton Down
Salisbury
Wiltshire SP4 0JG

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

Great Britain

06765853001

4. Title of the invention

Delivery of superoxide dismutase to neuronal cells

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

MATHYS & SQUIRE
100 Grays Inn Road
London WC1X 8AL

Patents ADP number *(if you know it)*

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DELIVERY OF SUPEROXIDE DISMUTASE TO NEURONAL CELLS

The present invention relates to compositions and methods for delivery of superoxide dismutase (SOD) to neuronal cells, and in particular for delivery of SOD to mitochondria in those cells. The present invention also provides methods of making the constructs.

A number of nervous system disorders would benefit considerably from rapid intervention with several therapeutic agents. Examples of such disorders are global, focal or spinal cord ischaemia induced by stroke or injury. Neurones injured by trauma or ischaemia produce elevated levels of transmitter substances which results in large high levels of reactive oxygen species. These, in high concentrations are toxic to the both neurones and surrounding cells which potentiates and amplifies the damage process. Rapid therapeutic intervention with agents to reduce oxidative stress in cases of neuronal injury caused by stroke or trauma could therefore significantly limit this secondary damage process. One such potential therapeutic agent is superoxide dismutase which neutralises the harmful effects of the superoxide radicals by converting them to hydrogen peroxide and oxygen.

Francis and co-workers demonstrated that post-ischaemic infusion of Copper/Zinc superoxide dismutase (Cu/Zn-SOD) reduces cerebral infarction following ischaemia/reperfusion in rats (Experimental Neurology (1997) 146, 435-443) through the reduction of damaging free-radical oxygen. Lim et al. have shown than administration of Cu/Zn-SOD attenuates the level of reperfusion injury following spinal cord ischaemia in dogs (Ann. Thorac. Surg. (1986) 42, 282-286). Cuevas et al. have similarly demonstrated protective effects of SOD, both on neurological recovery and spinal infarction, in ischaemic reperfusion injury of the rabbit spinal cord (Acta Anat. (1990) 137, 303-310. A major problem in the use of such therapies is the maintenance of useful concentration of the active agent at the site of trauma. Enzymes such as Cu/Zn-SOD are rapidly cleared from the systemic circulation; in the case of the latter enzyme the $t_{1/2}$ in rat is 4-8 minutes. A number

of strategies have been employed to overcome these difficulties. Matsumiaya et al. (Stroke (1991) 22, 1193-1200) conjugated Cu/Zn-SOD to polyethylene glycol to increase it half-life in the blood. Francis and co-workers (Experimental Neurology (1997) 146, 435-443) describe the neuronal delivery of Cn/Zn-SOD by fusion of the enzyme to the binding domain of tetanus toxin. None of these strategies, however, are particularly efficient at delivering the enzyme to the intracellular neuronal compartments where the enzyme can be effective.

In most eukaryotic species, two intracellular forms of superoxide dismutase exist: the Cu/Zn-SOD which is located within the cytoplasmic and nuclear compartments and manganese superoxide dismutase (Mn-SOD) which is located within the mitochondrial matrix. Human Mn-SOD is a tetrameric enzyme and is larger than the dimeric Cu/Zn-SOD. Several studies have shown that decreased Mn-SOD may be associated with one or more chronic diseases such as ovarian cancer (Nishida *et al.* (1995) Oncology Reports, 2,643-646) and diabetes (L'Abbe *et al.,* (1994) Proc Soc Exp Biol Med, 207, 206-274). In addition, mice in which the Mn-SOD gene has been knocked out exhibit several novel pathogenic phenotypes including severe anaemia, degeneration of neurones in the basal ganglia and brainstem, and progressive motor disturbances characterised by weakness and rapid fatigue (Lebovitz *et al.,* (1996) Proc Natl Acad Sci USA, 93, 9782-9787). In addition these mice showed extensive damage to the neuronal mitochondria. Over expression of Mn-SOD in cell lines and transgenic mice showed that damage and apoptosis of neurones under oxidative stress was markedly reduced (Keller *et al.*, (1998) Journal of Neuroscience, 18, 687-697). Mitochondrial damage was also reduced. These data showed that superoxide accumulation and subsequent mitochondrial damage play key roles in neuronal death induced by trauma both *in vitro* and *in vivo*. Delivery of agents which reduce the level of oxidative stress to neuronal cells may therefore reduce neuronal cell death and afford considerable therapeutic benefits.

Mn-SODs of bacterial origin such as that from *Bacillus stearothermophilus* or *Bacillus caldotenax* consist of two subunits and are smaller than the human isoform which is a tetramer. *B. stearothermophilus* and B. *caldotenax* Mn-SOD also have

onsiderably lower immunogenicity than the human isoform which is an advantage for continued therapeutic use. As enzymes for therapeutic applications, however, they suffer from similar drawbacks to other SODs in that very little of the administered enzyme is retained within the tissues where it would be therapeutically beneficial.

The botulinum neurotoxins are a family of seven structurally similar, yet antigenically different, protein toxins whose primary site of action is the neuromuscular junction where they block the release of the transmitter acetylcholine. The action of these toxins on the peripheral nervous system of man and animals results in the syndrome botulism, which is characterised by widespread flaccid muscular paralysis (Shone (1986) in 'Natural Toxicants in Foods', Editor D. Watson, Ellis Harwood, UK). Each of the botulinum neurotoxins consist of two disulphide-linked subunits; a 100 kDa heavy subunit which plays a role in the initial binding and internalisation of the neurotoxin into the nerve ending (Dolly et. al. (1984) Nature, 307, 457-460) and a 50 kDa light subunit which acts intracellularly to block the exocytosis process (McInnes and Dolly (1990) Febs Lett., 261, 323-326; de Paiva and Dolly (1990) Febs Lett., 277, 171-174). Thus it is the heavy chains of the botulinum neurotoxins that impart their remarkable neuronal specificity.

Tetanus toxin is structurally very similar to botulinum neurotoxins but its primary site of action is the central nervous system where it blocks the release of inhibitory neurotransmitters from central synapses (Renshaw cells). As described for the botulinum toxins above, it is domains within the heavy chain of tetanus toxin that bind to receptors on neuronal cells.

The binding and internalisation (translocation) functions of the clostridial neurotoxin (tetanus and botulinum) heavy chains can be assigned to at least two domains within their structures. The initial binding step is energy-independent and appears to be mediated by one or more domains within the H_c fragment of the neurotoxin (C-terminal fragment of approximately 50kDa) (Shone *et al.* (1985), Eur. J.

Biochem., 151, 75-82) while the translocation step is energy-dependent and appears to be mediated by one or more domains within the H_N fragment of the neurotoxin (N-terminal fragment of approximately 50kDa).

Isolated heavy chains are non-toxic compared to the native neurotoxins and yet retain the high affinity binding for neuronal cells. Tetanus and the botulinum neurotoxins from most of the seven serotypes, together with their derived heavy chains, have been shown to bind a wide variety of neuronal cell types with high affinities in the nM range (e.g botulinum type B neurotoxin; Evans *et al.* (1986) Eur. J. Biochem. 154, 409-416). Another key characteristic of the binding of the tetanus and botulinum heavy chains to neuronal cells is that the receptor ligand recognised by the various toxin serotypes differ. Thus for example, botulinum type A heavy chain binds to a different receptor to botulinum type F heavy chain and these two ligands are non-competitive with respect to their binding to neuronal cells (Wadsworth *et al.* (1990), Biochem J. 268, 123-128). Of the clostridial neurotoxin serotypes so far characterised (tetanus, botulinum A, B, C_1, D, E and F), all appear to recognise distinct receptor populations on neuronal cells. Collectively, the clostridial neurotoxin heavy chains provide high affinity binding ligands that recognise a whole family of receptors that are specific to neuronal cells.

It is an object of the invention to provide compositions and methods for delivery of SOD to neuronal cells. A further object is to provide compositions and methods for treatment or at least amelioration of conditions such as ischemic stroke.

Accordingly, the present invention provides a construct for delivering superoxide dismutase (SOD) to neuronal cells. In one aspect of the invention the construct consists of a SOD which has been combined with various functional protein domains to effect efficient targeting to the mitochondria within neuronal cells. The construct of specific embodiments of the invention, described in further detail below, contains the following elements:-

- a SOD which contains a leader sequence for targeting SOD to the mitochondria;
- a dimeric SOD which has low immunogenicity and high stability;
- a SOD which is linked to a domain that effects translocation across lipid membranes;
- a linkage between the SOD and the translocation domain that is cleaved within the neuronal cytosol; and
- a domain which selectively targets the construct to neuronal cells.

A first aspect of the invention thus provides a composition for delivery of superoxide dismutase (SOD) to neuronal cells, comprising:-

SOD; linked by a linker to

a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the SOD of the composition into the neuronal cell;

wherein after translocation of the SOD into the cell, the linker is cleaved to release SOD from the neuronal cell targeting domain.

By SOD is meant a sequence that has superoxide dismutase activity, and may also comprise a further sequence or sequences conferring additional properties on that portion of the constructs. For example, the SOD preferably also includes a sequence targeting the SOD to mitochondria in a neuronal cell.

The SOD may be a hybrid of Mn-SOD and a sequence targeting the hybrid to mitochondria. The SOD may be of bacterial or human origin. Alternatively, the SOD may be comprised of sequences from more than one origin, provided that it has superoxide dismutase activity.

The first domain may suitably be selected from (a) neuronal cell binding domains of clostridial toxins; and (b) fragments, variants and derivatives of the domains in

The second domain is suitably selected from (a) domains of clostridial or diphtheria toxins that translocate polypeptide sequences into cells, and (b) fragments, variants and derivatives of the domains of (a) that substantially retain the translocating activity of the domains of (a).

The linker of the construct is cleaved after translocation of the SOD into a neuronal cell so as to release SOD into the cell. A suitable linker is a disulphide bridge between cysteine residues, one residue on the SOD and one residue on the neuronal cell targeting domain, for example on the first sub-domain. Another example of a linker is a site for a protease found in neuronal cells.

In an embodiment of the invention a construct comprises SOD linked by a disulphide bridge to a neuronal cell targetting component comprising a first domain that binds to a neuronal cell and a second sub-domain that translocates the SOD into the neuronal cell. This construct is made recombinantly as a single polypeptide having a cysteine residue on the SOD which forms a disulphide bridge with a cysteine residue on the second domain. The SOD is covalently linked, initially, to the second domain. Following expression of this single polypeptide SOD is cleaved from the second domain leaving the SOD linked only by the disulphide bridge to the rest of the construct.

A second aspect of the invention provides a pharmaceutical compositions for treatment of oxidative damage to neuronal cells comprising a composition according to the invention with a pharmaceutically acceptable carrier. This composition may be used to deliver SOD to a neuronal cell, for example by administration of the composition by injection.

A third aspect of the invention provides a method of preparing a composition according to the invention, comprising chemically linking SOD, a linker and a neuronal cell targeting component. The SOD preferably is free of cysteine residues and the method preferably comprises treating the SOD with a cross-linker which will form a disulphide bridge with a cysteine residue on the neuronal cell targeting

domain.

In a further embodiment of the third aspect of the invention, there is provided a method of making a composition according to the invention comprising expressing a DNA that codes for a polypeptide having SOD, a linker, a neuronal cell targeting component. The polypeptide may further comprise a purification sequence and the method may further comprise purifying the polypeptide using this sequence and then cleaving the polypeptide to remove the purification sequence to leave SOD, the linker and the neuronal cell targeting component.

By virtue of the combination of properties defined above, constructs of the invention are surprisingly efficient at transporting SOD to the mitochondria within neuronal cells. The ability of the superoxide dismutase to be translocated into the cytosol by virtue of the 'translocation domain' within the construct and the cleavage of the enzyme from the latter domain within the cell is key to this targeting efficiency. As such the construct of the invention has considerable therapeutic value in treating neuronal diseases which results from oxidative stress and has several advantages over previously described SOD formulations. Mitochondria within cells containing high levels of superoxide radicals are particularly sensitive to damage and the ability of the construct of the invention to target the SOD to these organelles offers considerable advantage in that enzyme can act to remove the superoxide radicals where it is most required.

The construct of the invention may be used clinically in a variety of neuronal diseases which are caused or augmented by oxidative stress. Such conditions include ischaemic stoke, Parkinson's disease, Huntington's disease and motor neurone diseases. In the case of ischaemia/reperfusion injury caused by stroke or trauma, delivery of the construct of the invention to neurones of the hippocampus may afford considerable therapeutic benefits by reducing neuronal damage and death. Other neuronal diseases where the underlying cause is oxidative stress would also benefit from the therapeutic effects of the construct of the invention.

In the preferred aspect of the invention, the SOD is a dimeric, manganese superoxide dismutase (Mn-SOD) which is of bacterial origin and has low immunogenicity and high stability.

The use of a bacterial Mn-SOD in constructs has a number of advantages compared to the use of the human Mn-SOD isoform:-

- the low immunogenicity of the bacterial Mn-SOD is advantageous where repeated administration of the construct is required in which cases the induction of adverse host immune responses will be reduced; and

- the smaller size of constructs based on the dimeric bacterial Mn-SOD compared to human Mn-SOD (which is a tetramer) both reduces the likelihood of adverse immune responses and will increase the rate of diffusion of the construct to its target tissue.

In exercise of the invention, a bacterial Mn-SOD of low immunogenicity is derived from either *Bacillus stearothermophilus* (sequence as reported by Brock and Walker (1980) Biochemistry, 19, 2873-2882) or *Bacillus caldotenax* (gene and amino acid sequence as defined by Chambers *et al.,* (1992) FEMS Microbiology Letters, 91, 277-284) to which a mitochondrial leader sequence has been fused to the N-terminus of the protein by recombinant technology. This novel Mn-SOD is linked by a disulphide bridge to a translocation domain derived from a bacterial protein toxin, such as diphtheria toxin or botulinum neurotoxin. The translocation domain, in turn is fused to a receptor binding domain derived from a clostridial neurotoxin (botulinum or tetanus). The construct is produced initially as a single polypeptide by recombinant technology and subsequently converted to the construct of the invention by selective cleavage with a proteolytic enzyme. To produce the construct of the invention, a loop motif containing a unique protease site (e.g amino acid sequences specifically cleaved by proteases such as factor X, enterokinase, thrombin) and a cysteine residue is introduced between the C-terminus of the Mn-SOD and the N-terminus of the translocation domain such that

disulphide bridge is formed between the Mn-SOD and the translocation domain. Subsequent cleavage of the protease site generates the active construct. The final construct, when analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis in the presence of a reducing agent (e.g. dithiothreitol), dissociates into two major bands, one corresponding to the superoxide dismutase enzyme and a second corresponding to a polypeptide which contains the neuronal binding and translocation domains. In the absence of a reducing agent this dissociation does not occur and the complex is observed as a single major band on the gels.

Modification of Mn-SOD from *B. stearothermophilus* by addition of a mitochondrial targeting sequence offers several advantages over the use of human Mn-SOD which contains its own mitochondrial leader sequence. Firstly, the *Bacillus stearothermophilus* Mn-SOD has a high thermal stability and low immunogenicity which allows administration of several doses of the enzyme without provoking an immune response from the host that would reduces its efficacy. Secondly, the *Bacillus stearothermophilus* Mn-SOD is a small dimeric enzyme unlike the human Mn-SOD which is a tetramer. Recombinant constructs containing the latter enzyme would therefore have to be considerably larger and more complex in their structure.

In an embodiment of the invention, a DNA encoding a construct of the invention is made up by fusion of following DNA fragments commencing at the 5'end of the gene:-

an oligonucleotide encoding a modified human mitochondrial leader sequence (amino acid sequence: MLSRAVSGTSRQLAPALGYLGSRQ);

an oligonucleotide encoding Mn-SOD from *B. stearothermophilus* (coding for the amino acid sequence as defined in Brock and Walker (1980) Biochemistry, 19, 2873-2882);

an oligonucleotide encoding a linker peptide which contains the thrombin protease cleavage site and a cysteine residue for disulphide bridge formation

(peptide sequence: CGLVPAGSGP);

an oligonucleotide encoding a translocation domain derived from a botulinum neurotoxin (e.g. a DNA fragment coding for amino acid residues 449-871 of botulinum type A neurotoxin, or a DNA fragment coding for amino acid residues 441-858 of botulinum type B neurotoxin, or a DNA fragment coding for amino acid residues 440-864 of botulinum type F neurotoxin); and

an oligonucleotide encoding the receptor binding domain of a botulinum neurotoxin or tetanus neurotoxin (e.g. a DNA fragment coding for amino acid residues 872-1296 of botulinum type A neurotoxin, or a DNA fragment coding for amino acid residues 859-1291 of botulinum type B neurotoxin, or a DNA fragment coding for amino acid residues 865-1278 of botulinum type F neurotoxin, or a DNA fragment coding for amino acid residues 880-1315 of tetanus neurotoxin).

The above DNA fragments may be obtained and constructed by standard recombinant DNA methods. Expression and purification of the assembled construct may be obtained with a variety of suitable expression hosts, e.g. *Escherichia coli*, *Bacillus subtilis*.

Another DNA encoding the construct of the invention is made up the DNA fragments defined above in the previous embodiment but where an oligonucleotide encoding the translocation domain derived from diphtheria toxin (a DNA fragment coding for amino acid residues 194-386 of diphtheria toxin) replaces that for the translocation domain of the botulinum neurotoxin.

The translocation domain and neuronal binding domain of the construct may also be derived from any combination of different bacterial toxins. For example, the construct of the invention may contain a translocation domain derived from botulinum type F neurotoxin and a binding domain derived from botulinum type A neurotoxin.

A construct of the invention may be produced using protein chemistry techniques. Mn-SOD derived from *B. stearothermophilus* to which a mitochondrial leader sequence has been fused to the N-terminus of the protein by recombinant technology is modified with a heterobifunctional cross-linking reagent such as N-succinimidyl 3-[2-pyridyldithio] propionate (SPDP). The chemically modified enzyme is then combined to a cell targetting domain which contains the binding and translocation functional domains. The latter may be produced by recombinant technology or purified from the neurotoxins of *Clostridium botulinum* or *Clostridium tetani* by established methods. Chemical coupling of the SPDP-treated Mn-SOD may be accomplished using a free cysteine residue on the polypeptide containing the binding and translocation domains to give a construct of the invention.

Constructs of the invention may be introduced into either neuronal or non-neuronal tissue using methods known in the art. By subsequent specific binding to neuronal cell tissue, the targeted construct will exert it therapeutic effects. Alternatively, the construct may be injected near a site requiring therapeutic intervention, e.g. intrathecal or intracranial injection close to a site of trauma or disease.

The construct of the invention may also be administered with other agents which enhance its delivery to its target tissue. An example of such an agent is one which assists the passage of the construct of the invention through the blood-brain barrier to the central nervous system. The construct of the invention may also be administered in formulations with other therapeutic agents or drugs.

The dosage required for the construct of the invention will depend upon the application and could vary between 1μg/kg to100mg/kg of body weight.

The construct of the invention may be produced as a suspension, emulsion, solution or as a freeze dried powder depending on the application and properties of the release vehicle and its therapeutic contents. The construct of the invention may be resuspended or diluted in a variety of pharmaceutically acceptable liquids depending on the application.

"Clostridial neurotoxin" means a neurotoxin corresponding to tetanus neurotoxin or one of the seven botulinum neurotoxin serotypes (type A, B, C_1, D, E, F or G).

"Bind" in relation to the clostridial binding fragments, means the interaction between the clostridial fragment and one or more cell surface receptors or markers which results in localisation of the binding fragment or construct in the vicinity of the cell.

"Binding domain" of botulinum or tetanus neurotoxins means a domain of the toxin which retains the property of being a able to bind the receptors on neuronal cells in a similar manner to the intact neurotoxin and encompasses native domains and fragments, variants and derivatives that retain this binding function. This property of the binding domain can be assessed in competitive binding assays. In such assays, radiolabelled neurotoxin (e.g botulinum type A neurotoxin) is contacted with neuronal cells in the presence of various concentrations of non-radiolabelled fragment representing the 'binding domain' of the neurotoxin. The ligand mixture is incubated with the cells, at low temperature (0-3°C) to prevent ligand internalisation, during which competition between the radiolabelled neurotoxin and non-labelled 'binding domain' fragment may occur. In such assays when the unlabelled ligand used is binding domain of botulinum type A neurotoxin (residues 872-1296), the radiolabelled botulinum type A neurotoxin will be displaced from the neuronal cell receptors as the concentration of its non-labelled 'binding domain' is increased. The competition curve obtained in this case will therefore be representative of the behaviour of a 'binding domain' fragment being a able to bind the receptors on neuronal cells in a similar manner to the intact neurotoxin. This property of the binding domain may be used to identify other suitable protein domains which have the desired binding properties. Examples of binding domains derived from clostridial neurotoxins are as follows:-

Botulinum type A neurotoxin	– amino acid residues (872 - 1296)
Botulinum type B neurotoxin	– amino acid residues (859 - 1291)
Botulinum type C neurotoxin	– amino acid residues (867 - 1291)
Botulinum type D neurotoxin	– amino acid residues (863 - 1276)

Botulinum type E neurotoxin	– amino acid residues (846 - 1252)
Botulinum type F neurotoxin	– amino acid residues (865 - 1278)
Botulinum type G neurotoxin	– amino acid residues (864 - 1297)
Tetanus neurotoxin	– amino acid residues (880 - 1315)

"Translocation domain" means a domain or fragment of a protein which effects transport of itself and/or other proteins and substances across a membrane or lipid bilayer and encompasses native domains and fragments, variants and derivatives that retain this binding function. The latter membrane may be that of an endosome where translocation will occur during the process of receptor-mediated endocytosis. Translocation domains can frequently be identified by the property of being able to form measurable pores in lipid membranes at low pH (Shone *et al.* Eur J. Biochem. 167, 175-180). The latter property of translocation domains may thus be used to identify other protein domains which could function as the translocation domain within the construct of the invention. Examples of translocation domains derived from bacterial neurotoxins are as follows:-

Botulinum type A neurotoxin	– amino acid residues (449 - 871)
Botulinum type B neurotoxin	– amino acid residues (441 - 858)
Botulinum type C neurotoxin	– amino acid residues (442 - 866)
Botulinum type D neurotoxin	– amino acid residues (446 - 862)
Botulinum type E neurotoxin	– amino acid residues (423 - 845)
Botulinum type F neurotoxin	– amino acid residues (440 - 864)
Botulinum type G neurotoxin	– amino acid residues (442 - 863)
Diphtheria toxin	– amino acid residues (194 - 386)
Tetanus neurotoxin	– amino acid residues (458 - 879)

"Translocation" in relation to translocation domain, means the internalisation events which occur after modified clostridial binding fragments bind to the cell surface. These events lead to the transport of substances into the cytosol of neuronal cells.

"Unique protease site" means a protease site incorporated into the construct such that the molecule may be proteolysed at pre-determined sites by a selected

protease. The specificity of these proteases is such that cleavage to other parts of the construct does not occur. Examples of unique protease sites are the amino acid sequences cleaved by proteases such as: thrombin, factor X, enterokinase.

A fourth aspect of the invention provides a composition for delivery of a therapeutic agent to neuronal cells, comprising:-

the therapeutic agent; linked by a linker to
a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the therapeutic agent of the composition into the neuronal cell;

wherein after translocation of the therapeutic agent into the cell, the linker is cleaved to release the therapeutic agent from the neuronal cell targeting domain.

There now follows description of specific embodiments of the invention illustrated by drawings in which:-

Fig. 1 gives examples of novel Mn-SODs derived from *B. stearothermophilis* and *B. caldotenax*. Two examples of mitochondrial leader sequences are shown. In one example, a cysteine residue at position 7 has been mutated to a serine residue. This change enables the production of the construct of the invention without the formation of disulphide bridges in undesirable positions;

Fig. 2 gives the amino acid and gene sequences of novel bacterial Mn-SODs derived from *B. stearothermophilis* and *B. caldotenax* and containing a modified mitochondrial leader (targeting) sequences;

Figure 3 gives examples of novel Mn-SOD fusion proteins showing the use of peptides and proteins to facilitate purification of the enzyme from the production strain. Various protein and peptide tags (such as histidine-6, S-peptide, maltose-

inding protein, calmodulin-binding protein) may be fused to the Mn-SOD to allow rapid purification by affinity chromatography methods. Unique protease sites are incorporated between the purification tag and the Mn-SOD to enable removal of the tag after purification. Protein and peptide tags may be removed by treatment of the fusion protein with the relevant specific protease (e.g. factor X, thrombin, enterokinase);

Fig. 4 gives an example recombinant Mn-SOD construct of the invention. From the N-terminus of the protein, the construct consists of the following components:- (1) a mitochondrial leader (targeting) sequence, (2) a Mn-superoxide dismutase, (3) a loop which contains a unique protease site and which allows disulphide bridge formation, (4) a translocation domain, (5) a neuronal targeting domain. The construct is produced as a single polypeptide; subsequent cleavage with a protease specific for the 'unique protease site' contained within the loop region generates the di-chain construct. Purification tags could added to the constructs as exemplified in Figure 3;

Fig. 5 shows the complete amino acid sequences of examples of a recombinant Mn-SOD constructs;

Fig. 6 shows the production of a Mn-SOD construct by chemical methods. The method uses a recombinant Mn-SOD, purified as described in Example 1 and coupled to a polypeptide containing the translocation and binding domains as described in Example 4.

Example 1.

Production and purification of novel *Bacillus stearothermophilus* Mn-SOD containing a mitochondrial leader sequence.

Standard molecular biology protocols were used for all genetic manipulations (*eg.* Sambrook *et al.* 1989, Molecular Cloning a Laboratory Manual, Second Edition,

Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). A synthetic gene encoding the mitochondrial targeting sequence (amino acids 1-27) of the human Mn-SOD gene was cloned as an *Nde*I - *Nru*I fragment into an expression vector so that the transcriptional start corresponds to the ATG codon within the *Nde*I site. The Mn-SOD gene from *B. stearothermophilus* or *B. caldotenax* was amplified using PCR to give a blunt *Pvu*II site corresponding to the leucine amino acid at position 5 at the 5' end, and a *Bam*HI site outside the stop codon at the 3' end. This *Pvu*II-*Bam*HI fragment was cloned into the expression vector carrying the mitochondrial targeting sequence (digested *Nru*I-*Bam*HI) to generate "in-frame" gene fusions. In addition to the wild type mitochondrial targeting sequence, a variant was constructed in which the cysteine at position 7 was changed to serine

The recombinant Mn-SOD expressed in pET28 were produced with amino-terminal histidine (6 His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Ni^{2+} charged column or an anti-T7 immunoaffinity column (Smith *et al.* 1988, *Journal of Biological Chemistry*, 263: 7211-7215). Incorporation of a factor X protease cleavage between the peptide tag and the expressed Mn-SOD allowed this to be removed after purification. Briefly, cultures of *E.coli* BL21 (DE3) pET28-Mn-SOD were grown in Terrific broth-kanamycin (30 μgml^{-1}) to an OD_{600} nm of 2.0, and protein expression was induced by the addition of 500μM IPTG for approximately 2 h. Cells were lysed by freeze/thaw followed by sonication, lysates cleared by centrifugation and supernatants loaded onto an anion exchange column (MonoQ™ column on a Fast Protein Liquid Chromatography system; Pharmacia Biotech, Uppsala, Sweden). Eluted recombinant Mn-SOD was then desalted and further purified by affinity chromatography on a chelating sepharose column charged with Ni^{2+} (Pharmacia Biotech, Uppsala, Sweden). After loading proteins onto the column and subsequent washing, the purified Mn-SOD was eluted with imidazole. All buffers used were as specified by the manufacturer.

A 'maltose binding protein' purification tag was also employed for the purification some batches of Mn-SOD. The use of this system is described in detail in New England Biolabs Instruction Manual "Protein Fusion and Purification System" (ver

3.02).

Other tags and protease cleavage site may also be incorporated into the sequence to facilitate purification of Mn-SOD as exemplified in Figure 3.

For purification of a novel *Bacillus stearothermophilus* Mn-SOD which was not conjugated to protein purification tag, the following procedure was used. After harvesting, cells were broken by high pressure homogenisation crude extracts were clarified by centrifugation and batch purified on DE-23 cellulose. The fraction eluted with 0.4M NaCl contained the Mn-SOD. This fraction was then further purified by various chromatographic media using the following sequence:-

DEAE-Sepharose ion exchange chromatography at pH 8.0; elution of the Mn-SOD with a NaCl gradient;

hydroxylapatite chromatography at pH 6.8; elution of Mn-SOD with a phosphate gradient at pH 6.8;

ion exchange chromatography on Q-Sepharose at pH 7.5; elution with a NaCl gradient; and

gel filtration on Sephacryl S-200.

The purified Mn-SOD may be dialysed against Hepes buffer (0.1M, pH7.4) containing 0.15M NaCl and stored at –80°C.

Example 2.

Preparation and purification of a recombinant Mn-SOD construct of the invention.

Standard molecular biology protocols were used for all genetic manipulations (*eg.* Sambrook *et al.* 1989, Molecular Cloning a Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Various DNA

fragments of the construct were generated using Recursive PCR reactions (Prodromou & Pearl 1992, *Protein Engineering*, 5: 827-829) using self-priming oligonucleotides containing the desired sequence. For the expression of clostridial neurotoxin fragments the codon bias and GC/AT base ratio was adjusted for ease of expression in *E. coli*. Fragments were cloned sequentially into pLitmus 38 (New England Biolabs, Inc., Beverly, MA) to assemble the entire gene. Constructs for expression were sub-cloned into pET28b (Novagen Inc., Madison, WI) replacing the *Eco*R1-*Hin*dIII fragment. The ligation reactions were transformed into *E.coli* DH5α (Life Technologies Inc., Gaithersburg, MD). Plasmid DNA was amplified, purified and screened for the presence of the appropriate sequence (Ausubel *et al.* 1989, Current Protocols in Molecular Biology, John Wiley & Sons, New York). Gene constructions confirmed as possessing the correct sequences were then transformed into the expression host *E. coli* BL21 (DE3) (Studier & Moffatt 1986, *Journal of Molecular Biology*, 189: 113-130).

The recombinant constructs expressed in pET28 were produced with amino-terminal histidine (6 His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Ni^{2+} charged column or an anti-T7 immunoaffinity column (Smith *et al.* 1988, *Journal of Biological Chemistry*, 263: 7211-7215). Incorporation of a factor X protease cleavage between the peptide tags and the expressed Mn-SOD Constructs allowed these to be removed after purification. Briefly, cultures of *E.coli* BL21 (DE3) pET28-Mn-SOD Construct were grown in Terrific broth-kanamycin (30 μgml^{-1}) to an OD_{600} nm of 2.0, and protein expression was induced by the addition of 500μM IPTG for approximately 2 h. Cells were lysed by freeze/thaw followed by sonication, lysates cleared by centrifugation and supernatants loaded onto an anion exchange column (MonoQ™ column on a Fast Protein Liquid Chromatography system; Pharmacia Biotech, Uppsala, Sweden). Eluted recombinant Mn-SOD Construct was then desalted and further purified by affinity chromatography on a chelating sepharose column charged with Ni^{2+} (Pharmacia Biotech, Uppsala, Sweden). After loading proteins onto the column and subsequent washing, the purified Construct was eluted with imidazole. All buffers used were as specified by the manufacturer.

A 'maltose binding protein' purification tag was also employed for the purification some batches of Mn-SOD Constructs. The use of this system is described in detail in New England Biolabs Instruction Manual "Protein Fusion and Purification System" (ver 3.02).

It would also be evident to anyone skilled in the art that other tags and protease cleavage site may also be incorporated into the sequence to facilitate purification as exemplified in Figure 3.

The amino sequences of several recombinant Mn-SOD constructs are shown in Figure 5.

Example 3.

Preparation of botulinum heavy chains by chemical methods.

The various serotypes of the clostridial neurotoxins may be prepared and purified from various toxigenic strains of *Clostridium botulinum* and *Clostridium tetani* by methods employing standard protein purification techniques as described previously (Shone and Tranter 1995, Current Topics in Microbiology, 194, 143-160; Springer). Samples of botulinum neurotoxin (1mg/ml) are dialysed against a buffer containing 50mM Tris-HCl pH 8.0, 1M NaCl and 2.5M urea for at least 4 hours at 4°C and then made 100mM with dithiothreitol and incubated for 16h at 22°C. The cloudy solution which contains precipitated light chain is then centrifuged at 15000 x g for 2 minutes and the supernatant fluid containing the heavy chain retained and dialysed against 50mM Hepes pH 7.5 containing 0.2M NaCl and 5mM dithiothreitol for at least 4 hours at 4°C. The dialysed heavy chain is centrifuged at 15000 x g for 2 minutes and the supernatant retained and dialysed thoroughly against 50mM Hepes pH 7.5 buffer containing 0.2M NaCl and stored at -70°C. The latter procedure yields heavy chain >95% pure with a free cysteine residue which can be used for chemical coupling purposes. Biological (binding) activity of the heavy chain may be assayed as described in Example 5.

The heavy chains of the botulinum neurotoxins may also be produced by chromatography on QAE Sephadex as described by the methods in Shone and Tranter (1995) (Current Topics in Microbiology, 194, 143-160; Springer).

Example 4

Production of Mn-SOD constructs by chemical methods

Bacillus stearothermophilus Mn-SOD fused to a mitochondrial leader sequence was purified as described in Example 1. The Mn-SOD was chemically modified by treatment with a 3-5 molar excess of N-succinimidyl 3-[2-pyridyldithio] propionate (SPDP) in 0.05M Hepes buffer pH 7.0 containing 0.1M NaCl for 60 min at 22°C. The excess SPDP was removed by dialysis against the same buffer at 4°C for 16h. The substituted SOD was then mixed in a 1:1 molar ratio with heavy chain purified from *Clostridium botulinum* type A neurotoxin purified as described in Example 3 and incubated at 4°C for 16h. During the incubation period the Mn-SOD was conjugated to the botulinum heavy chain fragment by free sulphydryl groups (see Figure 4). After incubation, the Mn-SOD-construct was purified by gel filtration chromatography on Sephadex G200.

Constructs of the invention may also be formed by the above method using polypeptides containing the translocation and binding domains that have been produced by recombinant technology as outlined in Example 2.

Example 5.

Assay of the biological activity of constructs -
demonstration of high affinity binding to neuronal cells.

Clostridial neurotoxins may be labelled with 125-iodine using chloramine-T and its binding to various cells assessed by standard methods such as described in Evans *et al.* 1986, Eur J. Biochem., 154, 409 or Wadsworth *et al.* 1990, Biochem. J.

68, 123). In these experiments the ability of Mn-SOD constructs to compete with native clostridial neurotoxins for receptors present on neuronal cells or brain synaptosomes was assessed. All binding experiments were carried out in binding buffers. For the botulinum neurotoxins this buffer consisted of: 50mM hepes pH 7.0, 30mM NaCl, 0.25% sucrose, 0.25% bovine serum albumin. For tetanus toxin, the binding buffer was: 0.05M tris-acetate pH 6.0 containing 0.6% bovine serum albumin. In a typical binding experiment the radiolabelled clostridial neurotoxin was held at a fixed concentration of between 1-20nM. Reaction mixtures were prepared by mixing the radiolabelled toxin with various concentrations of unlabelled neurotoxin or construct. The reaction mixture were then added to neuronal cells or rat brain synaptosomes and then incubated at 0-3°C for 2hr. After this period the neuronal cells of synaptosomes were washed twice with binding ice-cold binding buffer and the amount of labelled clostridial neurotoxin bound to cells or synaptosomes was assessed by γ-counting. In an experiment using an Mn-SOD construct which contained the binding domain from botulinum type A neurotoxin, the construct was found to compete with ^{125}I-labelled botulinum type A neurotoxin for neuronal cell receptors in a similar manner to unlabelled native botulinum type A neurotoxin. These data showed that the construct had retained binding properties of the native neurotoxin.

Example 6

Assay of the biological activity of constructs – measurement of the Mn-SOD activity.

Mn-SOD activity in samples and constructs was measured by a modification (Brehm *et al.* (1991) Appl. Microbiol. Biotechnol., 36,358-363) of the procedure described by McCord and Fridovich (J. Biol. Chem. (1969), 244, 6049-6055). Aliquots (20μl) of samples or constructs containing Mn-SOD were added to 1ml of 0.05M potassium phosphate buffer pH 7.5 containing 1 x 10^{-4} M EDTA, 2.5 x 10^{-5}M ferricytochrome C and 7 x 10^{-3}M sodium xanthine in a thermostatted cuvette at 30°C. Sufficient xanthine oxidase was added to produce a rate of reduction of the

ferricytochrome C at 550nm of approx. 0.1 absorbance units/minute in the absence of Mn-SOD. Under these conditions the amount Mn-SOD that was required to reduce the rate of reduction of ferricytochrome C by 50% was defined as one unit of activity.

Using such assays the Mn-SOD activity within constructs was assessed.

Examples 7.

Demonstration of the targeting of Mn-SOD to the mitochondria of neuronal cells by constructs of the invention

Mn-SOD construct containing the translocation and targeting domains derived from botulinum type A neurotoxin was incubated at various concentrations (0.01-10μM final concentration) with a neuroblastoma cell line NG108. Incubations were carried out over a 6h period or overnight at 37°C. In some experiments, construct radiolabelled with ^{125}iodine was used. After incubation with the construct cells, were removed from culture flasks by gentle scraping and centrifuged at 200 x g. Cells were then resuspended in breaking buffer (0.6M mannitol, 20mM hepes pH 7.4 and 1mM phenylmethylsulphonyl chloride) and homogenised in a Dounce homogeniser. The homogenate was centrifuged at 200 x g for 5min and then the supernatant fluid recovered and centrifuged at 8000 x g for 10min. The 200 x g pellet (nuclear fraction) were pooled and resuspended in phosphate buffered saline. The 8000 x g pellets (mitochondrial fraction) were also pooled and resuspended in phosphate buffered saline. The supernatant fluid was saved and used to represent the cytosolic fraction.

Analysis of the sub-cellular distribution of Mn-SOD was carried out by Western blot analysis and, where radiolabelled construct was used, by analysis of the ^{125}I-labelled construct components by γ-counting and by autoradiography of cell fractions which had been separated by electrophoresis on SDS-polyacrylamide gels. For Western blot analysis, proteins in the cell fractions were separated by

lectrophoreses on SDS-polyacrylamide gels and then transferred to nitrocellulose membrane as described previously (Towbin *et al.* Proc.(1979) Natl. Acad. Sci. USA, 76, 4350). The presence of Mn-SOD in protein bands on nitrocellulose membranes was assessed by incubation with rabbit anti- Mn-SOD antibody followed by washing and incubation with anti-rabbit peroxidase conjugate. Addition of peroxidase substrates (3,3',5,5'- tetramethyl benzidine and H_2O_2) allowed visualisation and quantitation of the Mn-SOD in the various sub-cellular protein fractions. An enhanced chemiluminescence system (Amersham International) was also used in some experiments to increase the sensitivity.

Using the above assays systems, various Mn-SOD constructs of the invention were found to give significantly enhanced localisation of the Mn-SOD within the mitochondrial fraction of neuronal cells compared to control experiments which used native, unconjugated Mn-SOD from *B. stearothermoplilus*.

Example 8.

Formulation of the Mn-SOD construct for clinical use.

In a formulation of the Mn-SOD construct for clinical use, recombinant Mn-SOD construct would be prepared under current Good Manufacturing Procedures. The construct would be transferred, by dialysis, to a solution to give the product stability during freeze-drying. Such a formulation may contain Mn-SOD construct (10 mg/ml) in 5mM Hepes buffer (pH 7.2), 50mM NaCl, 1% lactose. The solution, after sterile filtration, would be aliquotted, freeze-dried and stored under nitrogen at -20°C.

Example 9.

Use of an Mn-SOD construct to treat stroke.

In a typical case of a middle aged or elderly man diagnosed as suffering from stroke, treatment with an Mn-SOD construct would begin immediately, ideally within 6 hours of the stroke occurring. Doses of the Mn-SOD construct (e.g. 100mg) reconstituted in a sterile saline solution would be administered intravenously. Further doses of the construct would be administered daily for 5-10 days. Such a patient would be expected to display reduced levels of ischaemia/reperfusion damage as assessed by magnetic resonance imaging compared to a similarly affected patient receiving no treatment. Relative improvements to muscle strength and co-ordination (MRC motor score) would be expected to be observed over the subsequent 12 month period.

The invention thus provides constructs and methods for delivery of SOD to neuronal cells.

CLAIMS

1. A composition for delivery of superoxide dismutase (SOD) to neuronal cells, comprising:-

SOD; linked by a linker to

a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the SOD of the composition into the neuronal cell;

wherein after translocation of the SOD into the cell, the linker is cleaved to release SOD from the neuronal cell targeting domain.

2. A composition according to Claim 1 for delivery of SOD to mitochondria of neuronal cells wherein the SOD comprises a sequence targeting the SOD to mitochondria in the neuronal cell.

3. A composition according to Claim 2 wherein the SOD is a hybrid of Mn-SOD and a sequence targeting the hybrid to mitochondria.

4. A composition according to Claim 2 or 3 wherein the mitochondria targeting sequence is derived from human Mn-SOD.

5. A composition according to any of Claims 1-4 wherein the SOD is bacterial SOD or is derived therefrom.

6. A composition according to any of Claims 1 to 5 wherein the first domain is selected from (a) neuronal cell binding domains of clostridial toxins; and (b) fragments, variants and derivatives of the domains in (a) that substantially retain the neuronal cell binding activity of the domains of (a).

7. A composition according to any Claims 1 to 6 wherein the second domain is selected from (a) domains of clostridial or diphtheria toxins that translocate polypeptide sequences into cells, and (b) fragments, variants and derivatives of the domains of (a) that substantially retain the translocating activity of the domains of (a).

8. A composition according to any of Claims 1 to 7 wherein the linker is a disulphide bridge.

9. A pharmaceutical composition for treatment of oxidative damage to neuronal cells comprising a composition according to any of Claims 1 to 8 and a pharmaceutically acceptable carrier.

10. A method of delivering SOD to a neuronal cell comprising administering a composition according to Claim 9.

11. A method according to Claim 10 comprising injecting the composition.

12. A method of making a composition according to any of Claims 1 to 8 comprising chemically linking SOD, a linker and a neuronal cell targeting domain.

13. A method of making a composition according to any of Claims 1 to 8 comprising expressing a DNA that codes for a polypeptide having SOD, a linker, and a neuronal cell targeting component.

14. A method according to claim 13 wherein the polypeptide further comprises a purification sequence and the method further comprises purifying the polypeptide and then cleaving the polypeptide to remove the purification sequence to leave SOD, the linker and the neuronal cell targeting component.

5. A composition for delivery of a therapeutic agent to neuronal cells, comprising:-

the therapeutic agent; linked by a linker to
a neuronal cell targeting component, comprising a first domain that binds to a neuronal cell and a second domain that translocates the therapeutic agent of the composition into the neuronal cell;

wherein after translocation of the therapeutic agent into the cell, the linker is cleaved to release the therapeutic agent from the neuronal cell targeting domain.

Figure 1. Examples of Novel Mn-Superoxide Dismutases

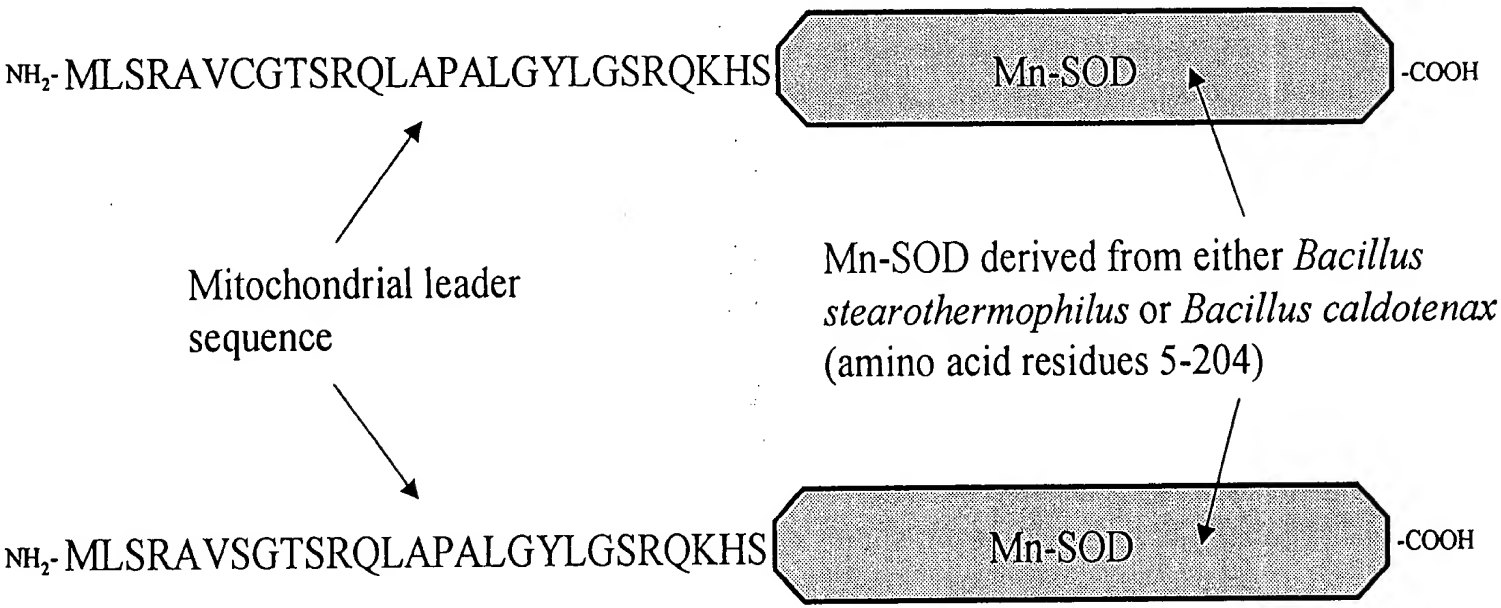


Figure 2. Amino acid and nucleotide sequences of novel Mn-superoxide dismutases derived from *Bacillus stearothermophilus* and *Bacillus caldotenax* enzymes

Novel Mn-superoxide dismutase based from *Bacillus stearothermophilus* Mn-SOD

MLSRAVSGTSRQLAPALGYLGSRQKHSLPALPYPYDALEPHIDK
ETMNIHHTKHHNTYVTNLNAALEGHPDLQNKSLEELLSNLEALP
ESIRTAVRNNGGGHANHSLFWTILSPNGGGEPTGELADAINKKF
GSFTAFKDEFSKAAAGRFGSGWAWLVVNNGELEITSTPNQDSPI
MEGKTPILGLDVWEHAYYLKYQNRRPEYIAAFWNVVNWDEVA
KRYSEAKAK

ATG CTG TCT CGT GCT GTT TCC GGT ACC TCT CGT CAG CTC GCT CCG GCT
CTG GGT TAC CTG GGT TCT CGT CAG AAA CAC TCG CTG CCA GCA TTG
CCG TAT CCG TAT GAT GCT CTG GAG CCG CAC ATC GAC AAA GAA ACG
ATG AAC ATT CAC CAC ACG AAG CAC CAT AAC ACA TAC GTT ACA AAT
TTG AAT GCG GCG CTT GAA GGA CAT CCG GAT TTG CAA AAC AAA TCG
CTC GAA GAA CTG CTC AGC AAT TTG GAA GCC CTT CCG GAA AGC ATC
CGC ACG GCG GTG CGC AAC AAC GGC GGC GGC CAT GCG AAC CAC TCG
CTT TTC TGG ACG ATT TTG TCG CCA AAT GGC GGC GGC GAG CCG ACG
GGT GAG CTG GCT GAC GCC ATC AAC AAA AAA TTC GGC AGC TTC ACC
GCG TTC AAA GAC GAG TTT TCG AAA GCA GCG GCC GGC CGT TTC GGT
TCC GGT TGG GCA TGG CTT GTT GTG AAC AAC GGC GAG CTG GAA ATC
ACA AGC ACG CCG AAC CAA GAT TCG CCG ATT ATG GAA GGC AAA ACG
CCG ATT CTC GGC TTG GAC GTT TGG GAG CAT GCG TAC TAC TTG AAA
TAC CAA AAC CGC CGT CCG GAA TAC ATT GCG GCA TTC TGG AAC GTC
GTC AAC TGG GAC GAA GTG GCG AAA CGG TAC AGC GAA GCG AAA GCA
AAA TAG

Figure 2 (continued)

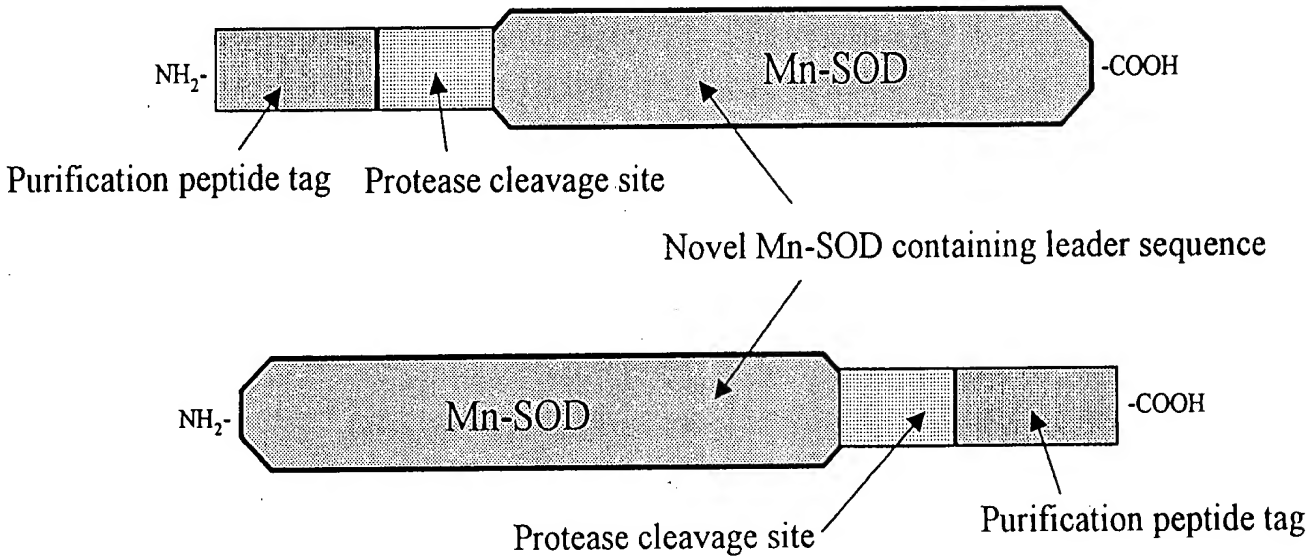
Novel Mn-Superoxide dismutase based on *Bacillus caldotenax* Mn-SOD

MLSRAVCGTSRQLAPALGYLGSRQKHSLPALPYPYDALEPHIDK
ETMNIHHTKHHNTYVTNLNAALEGHPDLQNKSLEELLSNLEALP
ESIRTAVRNNGGGHANHSLFWTILSPNGGGEPTGELAEAINKKF
GSFTAFKDEFSKAAAGRFGSGWAWLVVNNGELEITSTPNQDSPI
MEGKTPILGLDVWEHAYYLKYQNRRPEYIAAFWNIVNWDEVAK
RYSEAKAK

ATG CTG TCT CGT GCT GTT TGC GGT ACC TCT CGT CAG CTC GCT CCG GCT
CTG GGT TAC CTG GGT TCT CGT CAG AAA CAC TCG TTG CCA GCA TTG
CCG TAT CCG TAT GAT GCG CTT GAG CCG CAC ATC GAC AAA GAA ACG
ATG AAC ATT CAC CAC ACG AAG CAC CAT AAC ACA TAC GTT ACA AAT
TTG AAT GCG GCG CTT GAA GGG CAT CCG GAT TTG CAA AAC AAA TCG
CTC GAA GAA TTG CTC AGC AAT TTG GAA GCC CTT CCG GAA AGC ATT
CGC ACG GCG GTG CGC AAC AAC GGC GGC GGT CAT GCA AAC CAC TCG
CTT TTC TGG ACG ATT TTG TCG CCA AAT GGC GGC GGT GAG CCG ACG
GGT GAG CTG GCT GAG GCG ATC AAC AAA AAA TTC GGC AGC TTC ACC
GCG TTT AAA GAC GAG TTT TCG AAA GCA GCG GCC GGC CGT TTC GGT
TCT GGC TGG GCA TGG CTT GTC GTG AAC AAC GGC GAG CTG GAA ATT
ACG AGC ACG CCG AAC CAA GAC TCG CCG ATC ATG GAA GGC AAA ACG
CCG ATT CTC GGC TTG GAC GTT TGG GAG CAT GCG TAC TAC TTG AAA
TAC CAA AAC CGC CGT CCG GAA TAC ATT GCC GCA TTC TGG AAC ATT
GTC AAC TGG GAC GAA GTG GCG AAA CGG TAC AGC
GAA GCG AAA GCG AAG

Figure 3. Examples of a novel recombinant Mn-SOD fusion proteins showing positions of peptide tags and specific protease cleavage sites to facilitate purification of the enzyme.

By treatment with the appropriate protease, the purification peptide tags may be removed from the recombinant Mn-SOD.



Examples of purification peptides tags are:-

His6
S peptide
T7 peptide
Calmodulin binding peptide
Maltose binding protein

Examples of specific protease cleavage sites are:-

Thrombin
Enterokinase
Factor X

Figure 4. Recombinant Mn-Superoxide Dismutase Constructs

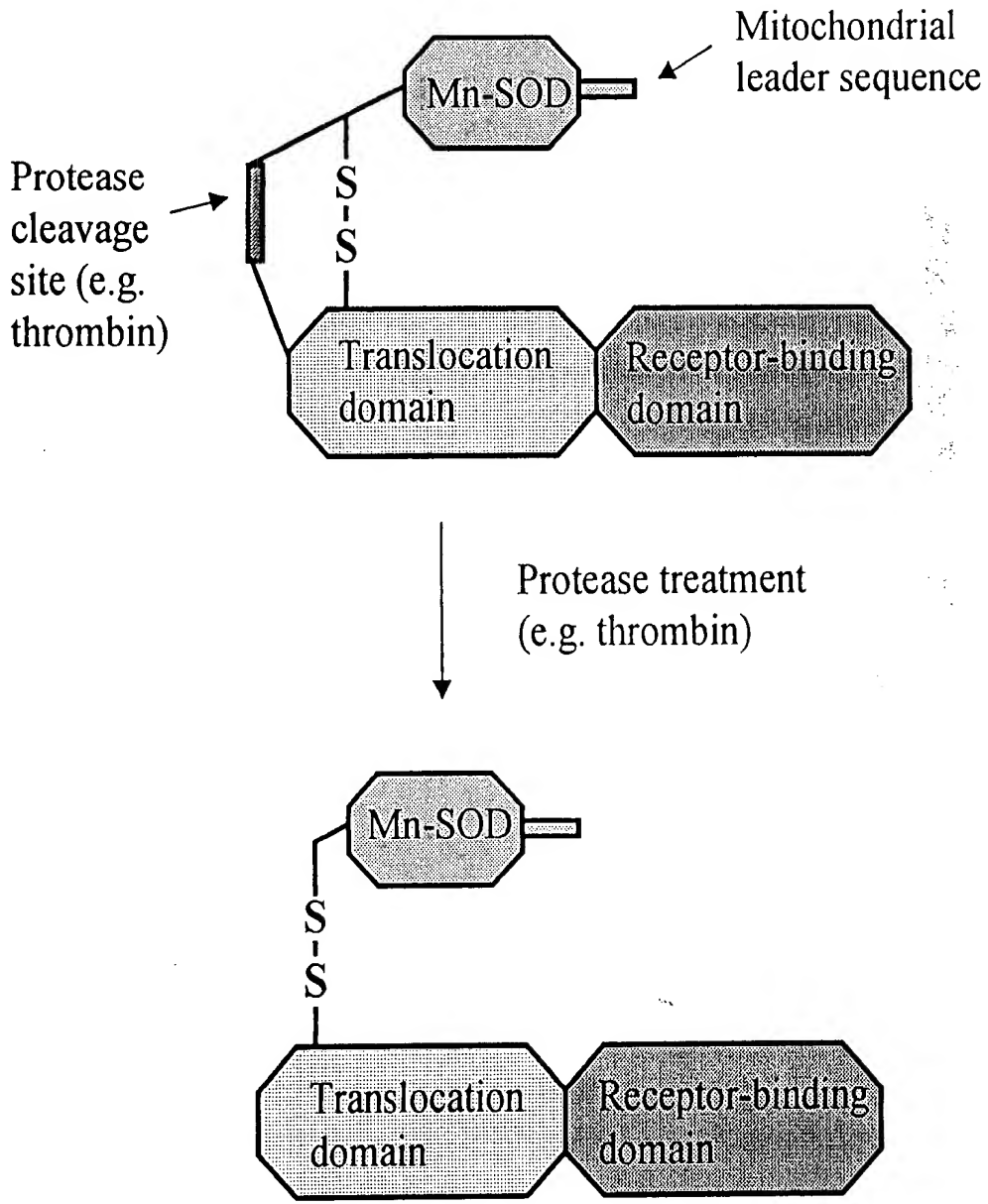


Figure 5. Examples of Amino Acid sequences of novel Mn-SOD constructs

(1) A Mn-superoxide construct containing:-
- a novel Mn-SOD (*Bacillus stearothermophilus*)
- a linker peptide containing a thrombin cleavage site
- a translocation domain derived from botulinum type A neurotoxin
- a receptor binding domain derived from botulinum type A neurotoxin

MLSRAVSGTSRQLAPALGYLGSRQKHSLPALPYPYDALEPHIDK
ETMNIHHTKHHNTYVTNLNAALEGHPDLQNKSLEELLSNLEALP
ESIRTAVRNNGGGHANHSLFWTILSPNGGGEPTGELADAINKKF
GSFTAFKDEFSKAAAGRFGSGWAWLVVNNGELEITSTPNQDSPI
MEGKTPILGLDVWEHAYYLKYQNRRPEYIAAFWNVVNWDEVA
KRYSEAKAKCGLVPAGSGPALNDLCIKVNNWDLFFSPSEDNFTN
DLNKGEEITSDTNIEAAEENISLDLIQQYYLTFNFDNEPENISIENL
SSDIIGQLELMPNIERFPNGKKYELDKYTMFHYLRAQEFEHGKS
RIALTNSVNEALLNPSRVYTFFSSDYVKKVNKATEAAMFLGWVE
QLVYDFTDETSEVSTTDKIADITIIIPYIGPALNIGNMLYKDDFVGA
LIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVLTVQTIDNALSKR
NEKWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKA
IINYQYNQYTEEEKNNINFNIDDLSSKLNESINKAMININKFLNQC
SVSYLMNSMIPYGVKRLEDFDASLKDALLKYIYDNRGTLIGQVD
RLKDKVNNTLSTDIPFQLSKYVDNQRLLSTFTEYIKNIINTSILNL
RYESNHLIDLSRYASKINIGSKVNFDPIDKNQIQLFNLESSKIEVIL
KNAIVYNSMYENFSTSFWIRIPKYFNSISLNNEYTIINCMENNSGW
KVSLNYGEIIWTLQDTQEIKQRVVFKYSQMINISDYINRWIFVTIT
NNRLNNSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHR
YIWIKYFNLFDKELNEKEIKDLYDNQSNSGILKDFWGDYLQYDK
PYYMLNLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYLNS
SLYRGTKFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATN
ASQAGVEKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQ
DNNGNDIGFIGFHQFNNIAKLVASNWYNRQIERSSRTLGCSWEFI
PVDDGWGERPL

Figure 5. (continued)

(2) A Mn-superoxide construct containing:-
- a novel Mn-SOD (*Bacillus stearothermophilus*)
- a linker peptide containing a thrombin cleavage site
- a translocation domain derived from botulinum type A neurotoxin
- a receptor binding domain derived from tetanus neurotoxin

MLSRAVSGTSRQLAPALGYLGSRQKHSLPALPYPYDALEPHIDK
ETMNIHHTKHHNTYVTNLNAALEGHPDLQNKSLEELLSNLEALP
ESIRTAVRNNGGGHANHSLFWTILSPNGGGEPTGELADAINKKF
GSFTAFKDEFSKAAAGRFGSGWAWLVVNNGELEITSTPNQDSPI
MEGKTPILGLDVWEHAYYLKYQNRRPEYIAAFWNVVNWDEVA
KRYSEAKAKCGLVPAGSGPALNDLCIKVNNWDLFFSPSEDNFTN
DLNKGEEITSDTNIEAAEENISLDLIQQYYLTFNFDNEPENISIENL
SSDIIGQLELMPNIERFPNGKKYELDKYTMFHYLRAQEFEHGKS
RIALTNSVNEALLNPSRVYTFFSSDYVKKVNKATEAAMFLGWVE
QLVYDFTDETSEVSTTDKIADITIIIPYIGPALNIGNMLYKDDFVGA
LIFSGAVILLEFIPEIAIPVLGTFALVSYIANKVLTVQTIDNALSKR
NEKWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKA
IINYQYNQYTEEEKNNINFNIDDLSSKLNESINKAMININKFLNQC
SVSYLMNSMIPYGVKRLEDFDASLKDALLKYIYDNRGTLIGQVD
RLKDKVNNTLSTDIPFQLSKYVDNQRLLSTFTEYIKILKKSTILNL
DINNDIISDISGFNSSVITYPDAQLVPGINGKAIHLVNNESSEVIVHK
AMDIEYNDMFNNFTVSFWLRVPKVSASHLEQYGTNEYSIISSMK
KHSLSIGSGWSVSLKGNNLIWTLKDSAGEVRQITFRDLPDKFNAY
LANKWVFITITNDRLSSANLYINGVLMGSAEITGLGAIREDNNITL
KLDRCNNNNQYVSIDKFRIFCKALNPKEIEKLYTSYLSITFLRDF
WGNPLRYDTEYYLIPVASSSKDVQLKNITDYMYLTNAPSYTNGK
LNIYYRRLYNGLKFIIKRYTPNNEIDSFVKSGDFIKLYVSYNNNEH
IVGYPKDGNAFNNLDRILRVGYNAPGIPLYKKMEAVKLRDLKTY
SVQLKLYDDKNASLGLVGTHNGQIGNDPNRDILIASNWYFNHLK
DKILGCDWYFVPTDEGWTND

Figure 5. (continued)

(3) A Mn-superoxide construct containing:-
- a novel Mn-SOD (*Bacillus caldotenax*)
- a linker peptide containing a thrombin cleavage site
- a translocation domain derived from botulinum type A neurotoxin
- a receptor binding domain derived from botulinum type F neurotoxin

MLSRAVCGTSRQLAPALGYLGSRQKHSLPALPYPYDALEPHIDK
ETMNIHHTKHHNTYVTNLNAALEGHPDLQNKSLEELLSNLEALP
ESIRTAVRNNGGGHANHSLFWTILSPNGGGEPTGELAEAINKKF
GSFTAFKDEFSKAAAGRFGSGWAWLVVNNGELEITSTPNQDSPI
MEGKTPILGLDVWEHAYYLKYQNRRPEYIAAFWNIVNWDEVAK
RYSEAKAKCGLVPAGSGPALNDLCIKVNNWDLFFSPSEDNFTND
LNKGEEITSDTNIEAAEENISLDLIQQYYLTFNFDNEPENISIENLSS
DIIGQLELMPNIERFPNGKKYELDKYTMFHYLRAQEFEHGKSRI
ALTNSVNEALLNPSRVYTFFSSDYVKKVNKATEAAMFLGWVEQL
VYDFTDETSEVSTTDKIADITIIIPYIGPALNIGNMLYKDDFVGALI
FSGAVILLEFIPEIAIPVLGTFALVSYIANKVLTVQTIDNALSKRNE
KWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQAEATKAIIN
YQYNQYTEEEKNNINFNIDDLSSKLNESINKAMININKFLNQCSVS
YLMNSMIPYGVKRLEDFDASLKDALLKYIYDNRGTLIGQVDRLK
DKVNNTLSTDIPFQLSKYVDNQRLLSTFTEYIKKIKDNSILDMRY
ENNKFIDISGYGSNISINGDVYIYSTNRNQFGIYSSKPSEVNIAQNN
DIIYNGRYQNFSISFWVRIPKYFNKVNLNNEYTIIDCIRNNNSGWK
ISLNYNKIIWTLQDTAGNNQKLVFNYTQMISISDYINKWIFVTITN
NRLGNSRIYINGNLIDEKSISNLGDIHVSDNILFKIVGCNDTRYVGI
RYFKVFDTELGKTEIETLYSDEPDPSILKDFWGNYLLYNKRYYLL
NLLRTDKSITQNSNFLNINQQRGVYQKPNIFSNTRLYTGVEVIIRK
NGSTDISNTDNFVRKNDLAYINVVDRDVEYRLYADISIAKPEKIIK
LIRTSNSNNSLGQIIVMDSIGNNCTMNFQNNNGGNIGLLGFHSNN
LVASSWYYNNIRKNTSSNGCFWSFISKEHGWQEN

Figure 5. (continued)

(4) A Mn-superoxide construct containing:-
- a novel Mn-SOD (*Bacillus stearothermophilus*)
- a linker peptide containing a thrombin cleavage site
- a translocation domain derived from diphtheria toxin
- a receptor binding domain derived from botulinum type A neurotoxin

MLSRAVSGTSRQLAPALGYLGSRQKHSLPALPYPYDALEPHIDK
ETMNIHHTKHHNTYVTNLNAALEGHPDLQNKSLEELLSNLEALP
ESIRTAVRNNGGGHANHSLFWTILSPNGGGEPTGELADAINKKF
GSFTAFKDEFSKAAAGRFGSGWAWLVVNNGELEITSTPNQDSPI
MEGKTPILGLDVWEHAYYLKYQNRRPEYIAAFWNVVNWDEVA
KRYSEAKAKCGLVPAGSGPSVGSSLSCINLDWDVIRDKTKTKIES
LKEHGPIKNKMSESPNKTVSEEKAKQYLEEFHQTALEHPELSEL
KTVTGTNPVFAGANYAAWAVNVAQVIDSETADNLEKTTAALSIL
PGIGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGELVDI
GFAAYNFVESIINLFQVVHNSYNRPAYSPGHKTNIINTSILNLRYES
NHLIDLSRYASKINIGSKVNFDPIDKNQIQLFNLESSKIEVILKNAI
VYNSMYENFSTSFWIRIPKYFNSISLNNEYTIINCMENNSGWKVSL
NYGEIIWTLQDTQEIKQRVVFKYSQMINISDYINRWIFVTITNNRL
NNSKIYINGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIK
YFNLFDKELNEKEIKDLYDNQSNSGILKDFWGDYLQYDKPYYML
NLYDPNKYVDVNNVGIRGYMYLKGPRGSVMTTNIYLNSSLYRGT
KFIIKKYASGNKDNIVRNNDRVYINVVVKNKEYRLATNASQAGV
EKILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGND
IGFIGFHQFNNIAKLVASNWYNRQIERSSRTLGCSWEFIPVDDGW
GERPL

Figure 5. (continued)

(5) A Mn-superoxide construct containing:-
- a novel Mn-SOD (*Bacillus stearothermophilus*)
- a linker peptide containing a Factor X cleavage site
- a translocation domain derived from diphtheria toxin
- a receptor binding domain derived from tetanus neurotoxin

MLSRAVSGTSRQLAPALGYLGSRQKHSLPALPYPYDALEPHIDK
ETMNIHHTKHHNTYVTNLNAALEGHPDLQNKSLEELLSNLEALP
ESIRTAVRNNGGGHANHSLFWTILSPNGGGEPTGELADAINKKF
GSFTAFKDEFSKAAAGRFGSGWAWLVVNNGELEITSTPNQDSPI
MEGKTPILGLDVWEHAYYLKYQNRRPEYIAAFWNVVNWDEVA
KRYSEAKAKCGIEGRAPGPSVGSSLSCINLDWDVIRDKTKTKIES
LKEHGPIKNKMSESPNKTVSEEKAKQYLEEFHQTALEHPELSEL
KTVTGTNPVFAGANYAAWAVNVAQVIDSETADNLEKTTAALSIL
PGIGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLVGELVDI
GFAAYNFVESIINLFQVVHNSYNRPAYSPGHKTILKKSTILNLDIN
NDIISDISGFNSSVITYPDAQLVPGINGKAIHLVNNESSEVIVHKAM
DIEYNDMFNNFTVSFWLRVPKVSASHLEQYGTNEYSIISSMKKHS
LSIGSGWSVSLKGNNLIWTLKDSAGEVRQITFRDLPDKFNAYLAN
KWVFITITNDRLSSANLYINGVLMGSAEITGLGAIREDNNITLKLD
RCNNNNQYVSIDKFRIFCKALNPKEIEKLYTSYLSITFLRDFWGN
PLRYDTEYYLIPVASSSKDVQLKNITDYMYLTNAPSYTNGKLNIY
YRRLYNGLKFIIKRYTPNNEIDSFVKSGDFIKLYVSYNNNEHIVGY
PKDGNAFNNLDRILRVGYNAPGIPLYKKMEAVKLRDLKTYSVQL
KLYDDKNASLGLVGTHNGQIGNDPNRDILIASNWYFNHLKDKIL
GCDWYFVPTDEGWTND

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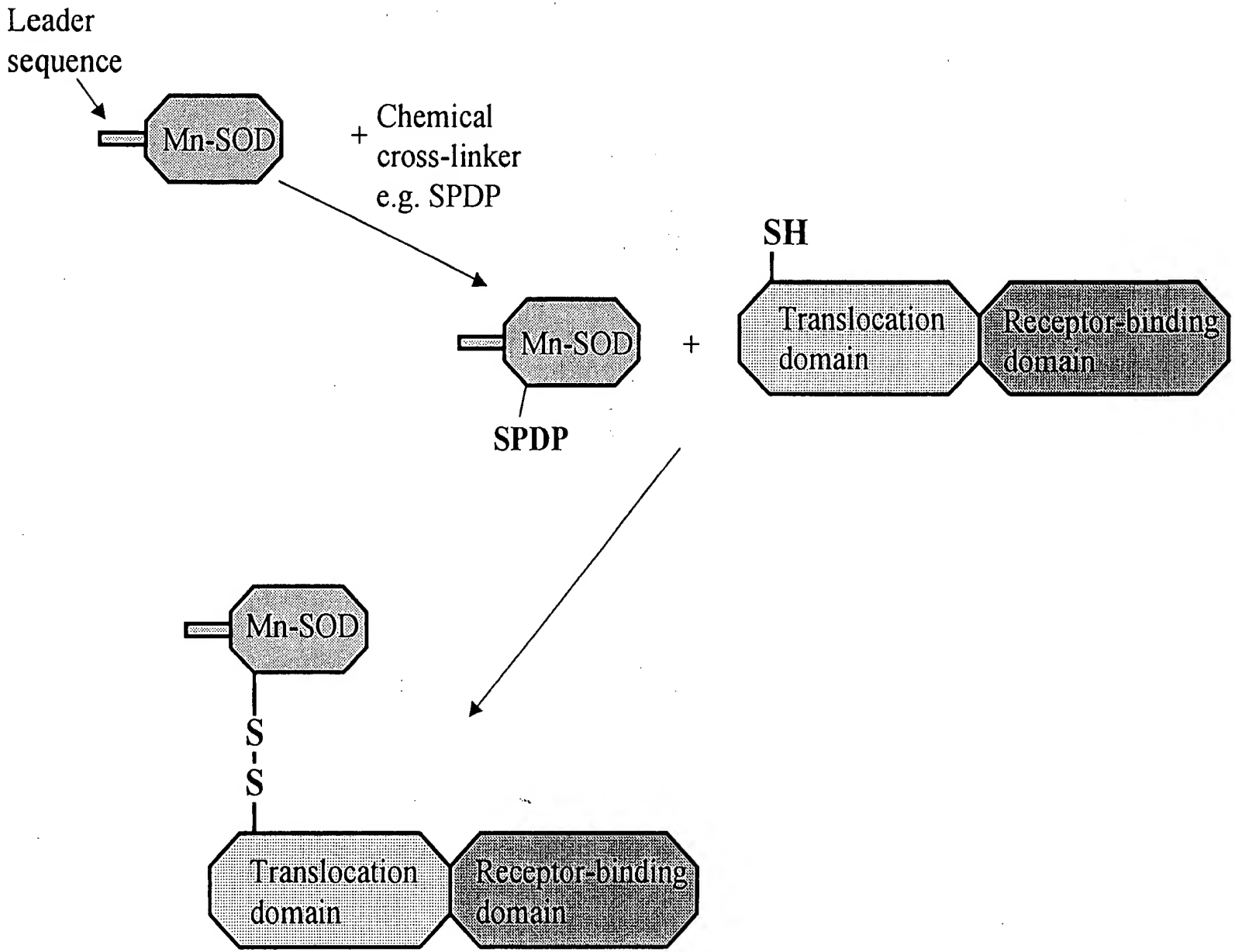
Figure 6. Production of Mn-Superoxide Dismutase Constructs by Chemical Conjugation



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